Marine Environmental Research 76 (2012) 41–47

Contents lists available at SciVerse ScienceDirect

Marine Environmental Research

journal homepage: www.elsevier.com/locate/marenvrev

Algal symbiont type affects gene expression in juveniles of the coral *Acropora tenuis* exposed to thermal stress

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ARTICLE INFO

Article history:
Received 3 August 2011
Accepted 12 September 2011

Keywords:
Acropora tenuis
Larvae
Thermal stress
Coral
Symbiodinium
Symbiosis
Gene expression
Fluorescent protein

ABSTRACT

Reef-building corals harbor symbiotic dinoflagellates, *Symbiodinium* spp., which are currently divided into several clades. The responses of corals associated with different *Symbiodinium* clades to thermal stress are not well understood, especially at a gene expression level. Juveniles of the coral *Acropora tenuis* inoculated with different algal types (clade A or D) were exposed to thermal stress and the expression levels of four putative stress-responsive genes, including genes coding green and red fluorescent proteins, an oxidative stress-responsive protein, and an ascorbic acid transporter, were analyzed by quantitative real-time PCR. The expression levels of the four genes decreased at high temperatures if juveniles were associated with clade A symbionts but increased if the symbionts were in clade D. The intensity of green fluorescence increased with temperature in clade D symbionts harboring juveniles, but not in juveniles associated with clade A symbionts. The present results suggest that genotypes of endosymbiotic algae affect the thermal stress responses of the coral juveniles.

1. Introduction

Coral reefs are experiencing increasing degrees of global and local environmental stresses (Hoegh-Guldberg, 1999; Hughes et al., 2003; Hoegh-Guldberg et al., 2007). Large-scale coral bleaching has been ascribed to increases in seawater temperature induced by climate change resulting from the greenhouse effect (Glynn, 1993; Crabbe, 2008). Coral bleaching is a breakdown of the symbiotic association between reef-building corals and their symbiotic algae, dinoflagellates of the genus *Symbiodinium*. *Symbiodinium* spp. consist of nine clades (A–I) (Baker, 2003; Coffroth and Santos, 2005; Pochon and Gates, 2010). Symbionts of clades A, B, C, and D are commonly associated with metazoan hosts (Baker, 2003), while clade C, E, F, G, H, and I are associated with large soritid foraminifera (Pochon and Pawlowski, 2006; Pochon and Gates, 2010). They are further divided into many subclades and strains (Coffroth and Santos, 2005).

Various clades of *Symbiodinium* show different levels of tolerance to stress and might be responsible for the differential tolerances of corals to bleaching stressors. The clade D *Symbiodinium* has been characterized as heat- or stress-tolerant based on its increased prevalence in the Caribbean and Indo-Pacific corals after bleaching events (Glynn et al., 2001; Toller et al., 2001; Baker et al., 2004; van Oppen et al., 2005; Jones et al., 2008). However, few studies have compared stress responses among corals hosting different *Symbiodinium* clades. In the high temperatures and turbid conditions of the north-eastern Indian Ocean, clade D *Symbiodinium* were more diverse and frequently observed than clade C in coral hosts (LaJeunesse et al., 2010). Colonies of *Acropora millepora* that had shuffled their dominant endosymbiont from C2 to D following bleaching exhibited higher photochemical efficiency and greater symbiont densities than C2-dominated colonies when exposed to heat stress (Berkelmans and van Oppen, 2006). On the other hand, *Acropora tenuis* juveniles associated with clade C1 symbionts showed much greater thermal tolerance than those associated with clade D symbionts based on photochemical efficiency measurements (Abrego et al., 2008). These results suggest that corals associated with different subclades of *Symbiodinium* might show different stress responses.

Changes in the expression patterns of stress-responsive genes are key indicators of the physiological response to stress and usually occur before metabolic or cellular damage becomes detectable. Recent studies have examined the gene expression patterns during thermal stress using cDNA microarrays as well as other gene expression analysis tools (Desalvo et al., 2008; Rodriguez-Lanetty et al., 2009; Voolstra et al., 2009). Heat shock

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doi:10.1016/j.marenvres.2011.09.004

proteins, superoxide dismutase, lectin, antioxidant proteins, and other genes have been identified as stress biomarkers in reef-building corals (Downs et al., 2000; Hashimoto et al., 2004; Csasezar et al., 2009; Vidal-Dupiol et al., 2009). Additionally, corals contain fluorescent proteins (FPs) (Dove et al., 2001), which have also been identified as stress response protein because the expression of a GFP-homolog was found to decrease under high temperatures (Smith-Keune and Dove, 2007). FPs also reduce the photoinhibitory effect of high levels of solar radiation, which in conjunction with thermal stress leads to bleaching (Salih et al., 2000). Finally, GFP might have the ability to quench reactive oxygen species (ROS) (Bou-Abdallah et al., 2006). FPs may therefore be possible indicators of the thermal stress within host coral cells.

While many researchers have reported molecular biomarkers of corals, few studies have examined stress responses of corals associated with different *Symbiodinium* clades at a molecular level (Desalvo et al., 2010). This may be in part due to the difficulty of maintaining corals associated with monoclonal *Symbiodinium* cells of different types for stress exposure experiments. We previously established a model system by infecting aposymbiotic *Acropora tenuis* primary polyps with a monoclonal population of cultured *Symbiodinium* cells (Yuyama et al., 2005). The objective of the present study was to compare the stress response of *A. tenuis* juveniles harboring different *Symbiodinium* clades. For this purpose, we infected aposymbiotic *A. tenuis* juveniles with a monoclonal population of cultured *Symbiodinium* cells. Two different *Symbiodinium* types (clades A and D) were used in this study, because we had difficulty to inoculate *A. tenuis* juvenile polyps with other cultures (clade B, F) or freshly isolated homologous *Symbiodinium* (clade C) (Yuyama et al., 2005, 2010). Changes in the gene expression patterns of FPs as well as FP content in juveniles were studied. Other stress-responsive proteins, including an oxidative stress-responsive protein and an ascorbic acid transporter, were also analyzed.

2. Materials and methods

2.1. Preparations of algae and coral juveniles

The monoclonal *Symbiodinium* strains PL-TS-1 (subclade A3) and CCMP 2556 (clade D) were obtained from the Bigelow Laboratory for Ocean Sciences (West Boothbay Harbor, ME, USA; https://ccmp.bigelow.org) and cultured in an IMK medium (Wako Chemicals, Osaka, Japan) at 25 °C under a 12-h light (100 μE m^{-2} s^{-1}):12-h dark cycle.

Colonies of *A. tenuis* were collected at the northern reef patch of Sesoko Island (26°39'48.3"N; 127°52'23.9"E), Okinawa, and kept in a running seawater tank at Sesoko Station, Tropical Biosphere Research Center, University of the Ryukyus (Okinawa, Japan) until spawning in June 2009. Two of five colonies spawned on 11 June. Eggs and sperms were mixed for fertilization and rinsed twice in filtered (0.22 μm) seawater (FSW) approximately 2 h after fertilization, after which they were kept in 2-L plastic containers (26 °C) (Harii et al., 2009). Seven days after fertilization, larvae were induced to metamorphose using Hym 248, a neuropeptide identified in Hydra (2 μM; Iwao et al., 2002). Approximately 50–100 larvae settled in two to three dishes. The FSW was replaced daily for both larvae and polyps.

2.2. Algal inoculation and stress treatment

Each *Symbiodinium* strain (2×10^3 cells·mL^{-1}) was introduced to primary polyps for 24 h. They were washed and maintained by changing the FSW daily at 26 °C until the number of symbionts in polyps increased. Two weeks after algal inoculation, the polyps were exposed to high temperature stress (32 °C) or control temperature (26 °C) under a 12-h dark: 12-h light (100 μE m^{-2} s^{-1}) cycle for 24 h. After 24 h treatment, algal density decreased in the polyps kept at high temperature (Fig. 1), indicating that the 24 h stress treatment was effective.

2.3. Count of Symbiodinium cells in polyps

Before and after stress treatment, three to five polyps from each treatment group were fixed and decalcified in 3% formaldehyde with 0.5M EDTA to estimate the algal density of the polyps. Following decalcification, polyps were homogenized, and the homogenate was diluted with a new fixation solution. The number of *Symbiodinium* cells in the dilute solution was counted using a hemocytometer under a florescence microscope (Optiphoto-2; Nikon, Tokyo, Japan).

2.4. Image analysis of fluorescence

Epifluorescence photomicrographs of juvenile polyps were taken under Multizoom AZ100 (Nikon) using a digital camera (Digital Sight DA-L1; Nikon). After the 12 h light period of the 24 h stress trestment, the polyps were moved to petri dishes (90 × 15 mm) containing filtered seawater and immediately observed. The intensity of green fluorescence within the polyp area was measured on digital micrograph images using the Photoshop (Adobe Systems, San Jose, CA, USA) and ImageJ software packages (Research Services Branch, National Institutes of Health, Bethesda, MD, USA). Green fluorescence was extracted using the green layer function of Photoshop and the average intensity of green fluorescence in the juveniles was calculated using the Mean Gray Value

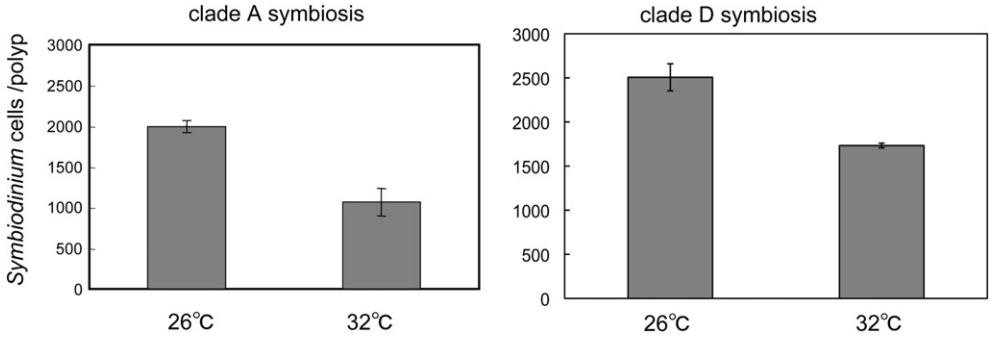

Fig. 1. Number of *Symbiodinium* cells per *Acropora tenuis* polyp of the control and high-temperature-treated groups. Polyps associated with clade A symbionts (left) or clade D symbionts (right) were kept at 26 °C or 32 °C for 24 h. Means ± SE ($n = 4$).

function of the ImageJ software. The intensity of the green fluorescence of each juvenile was calculated by multiplying the average fluorescence intensity by the area of the juvenile. The green fluorescence intensity before and after heat stress treatment was compared using Student's *t*-test (JMP 8.0; SAS Institute, Cary, NC, USA).

2.5. Quantitative real-time PCR

After stress treatment, all polyps were fixed in RNAlater (Ambion, Austin, TX, USA). Total RNA was extracted from approximately 40 mg of polyps in each treatment group using ISOGEN (Wako). First-strand cDNAs were synthesized from the 400 ng of total RNA using a QuantiTect Reverse Transcription Kit (Qiagen SpA, Milano, Italy). We selected four genes of interest from an EST library (Kii et al., 2007) and a high-quality cDNA expression profiling (HiCEP) library (Yuyama et al., 2010) of *A. tenuis* to investigate their expression analysis. Green fluorescent protein (GFP) and red fluorescent protein (RFP) coding genes were picked from the EST library and an oxidative stress-responsive protein and SLC23 genes were picked from the HiCEP library. The expression levels of these genes were investigated by real-time PCR, which was performed in triplicate using Fast SYBR Green Master Mix (Applied Biosystems) and the mean values were calculated. The data were analyzed using an ABI StepOne Plus™ Real-Time PCR System (Applied Biosystems). Primers for real-time PCR analyses of the genes were designed using Primer Express 3.0 (Applied Biosystems) (Table 1). Each real-time PCR reaction contained 10 μL of ABI universal PCR master mix, 1.5 mM forward primer, 1.5 mM reverse primer, and 2.0 μL cDNA template. The PCR conditions were 20 s denaturation at 95 °C, followed by 40 cycles of 3 s denaturation at 95 °C and 1 min annealing and extension at 60 °C. The data were normalized to the expression level of the ribosomal protein L5 (AtRibo-L5) gene of the coral (Yuyama et al., 2005).

3. Results

3.1. Changes in algal density and green fluorescence intensity

The numbers of algal cells in the polyps associated with clade A and clade D symbionts were 1994 ± 73 (mean ± SD, $n = 3$) and 2506 ± 153 ($n = 3$), respectively, 2 weeks after inoculation. The *Symbiodinium* cell density of the juveniles exposed to high temperature (32 °C) for 24 h was lower than the control juveniles by 46% and 31% in clade A symbiont- and clade D symbiont-harboring juveniles, respectively (Fig. 1).

Red, green, and blue fluorescences were observed in the polyps (Fig. 2). Given that the red fluorescence was mostly due to the chlorophyll fluorescence of *Symbiodinium* and the blue fluorescence was weak, we quantified the intensity of the green fluorescence of the polyps. The levels of green fluorescence intensity varied among individual polyps, but the average intensity of green fluorescence was higher in polyps associated with clade D symbionts than those associated with clade A symbionts at both temperatures (Student's *t*-test, $P < 0.05$) (Fig. 3). In clade D symbiont-associated polyps, the green fluorescence levels increased significantly after heat stress (Student's *t*-test, $P < 0.05$) whereas the green fluorescence levels of clade A symbiont-associated polyps did not change significantly. The fluorescence (green/red/blue) was also found in aposymbiotic polyps, although they seemed to be weak compared with symbiotic polyps (data not shown). We couldn't define the relationship between the localization of the symbiont and of coral fluorescence from photograph.

Table 1
Forward (F) and reverse (R) primers used in the quantitative real-time polymerase chain reaction assay to amplify 5 genes in the coral *Acropora tenuis*. Primer sequences and DDBJ/EMBL/Gene Bank accession numbers for genes of interest and internal control genes (Ribosomal L5) are also shown.

Gene	Accession No.	Primer (5′–3′)
GFP	AY646066	(F)TTGGCCAAAGTGCAAAAGG
		(R)ATGAGCCGCAGCATGTTCT
RFP	AB626607	(F)ACCGGATGGAAGGGTGTGT
		(R)GCCGTGGCCCGTGAT
Oxidative stress-responsive protein	DC999943	(F)TGACACCACCCCTGAGGAA
		(R)GCTTGGGAATGTAAAGCAACTGA
Solute carrier family 23	C1999915	(F)GCATCGCCTCAGTAAATTCAAA
		(R)TGTCGTATGGATAAGGCAGGAA
Ribosomal L5	AB222848	(F)CCATTGTAAATCTCATGCAGATTCA
		(R)TCCTTGCACCTTCAACAGAACAT

3.2. Gene expression analyses by quantitative real-time PCR

The full-length open reading frames of the GFP- and RFP-homologs of *A. tenuis* planula larvae were determined from their EST sequences (Kii et al., 2007) using the 5′- and 3′-RACE method. A search of the DDBJ (http://www.ddbj.nig.ac.jp/search/blast-j.html) database using BLASTx showed that the GFP-homolog was the closest match (E-value e^{-122}, 91% identity) to the homolog of *Acropora millepora* (Accession No. B5T1L4), and the RFP-homolog was the closest match (E-value e^{-124}, 90% identity) to RFP-homolog of *A. millepora* (Accession No. Q66PV2). The expression level of the GFP- and RFP-homolog decreased after high-temperature treatment in polyps associated with the clade A symbiont, while they increased in polyps associated with clade D symbiont (Fig. 4A). The gene expression levels of oxidative stress-responsive protein and an SLC23-homolog decreased at high temperature in polyps associated with clade A symbionts, while they increased in polyps associated with the clade D symbionts (Fig. 4B, C).

4. Discussion

Knowledge of how the symbiont type influences the stress response of the host or holobiont is essential for an understanding of how corals associated with diverse symbiont types respond to global environmental changes. However, few studies have examined the stress responses of a coral associated with different symbiont types at the gene expression level. Recently, Desalvo et al. (2010) conducted stress experiments using fragments from a single colony of *Montastraea faveolata* containing different symbiont types and found that the host transcriptomic states were more highly correlated with symbiont type than thermal stress. Maintaining adult colonies associated with a known monoclonal population of *Symbiodinium* is generally difficult because, in many corals, single colonies harbor different types of *Symbiodinium*. Voolstra et al. (2009) studied gene expression in coral larvae of *Acropora palmata* and *M. faveolata* after exposure to *Symbiodinium* strains with different compatibilities. They found that the host transcriptome remained almost unchanged when colonies were inoculated with competent symbionts, whereas extensive transcriptomic changes were reported for symbionts that failed to establish symbioses. Here, we inoculated juvenile polyps of *A. tenuis* with monoclonal populations of clade A and D *Symbiodinium* and compared the expression responses of four stress-related genes to thermal stress. Since *A. tenuis* adult colonies may have multiple types of *Symbiodinium*, juvenile polyps inoculated with monoclonal *Symbiodinium* provide effective experimental system to study how symbiont clade affects stress response of the host coral. This is the first study to show that different symbiont types modulate the host stress response differently at the gene expression level.

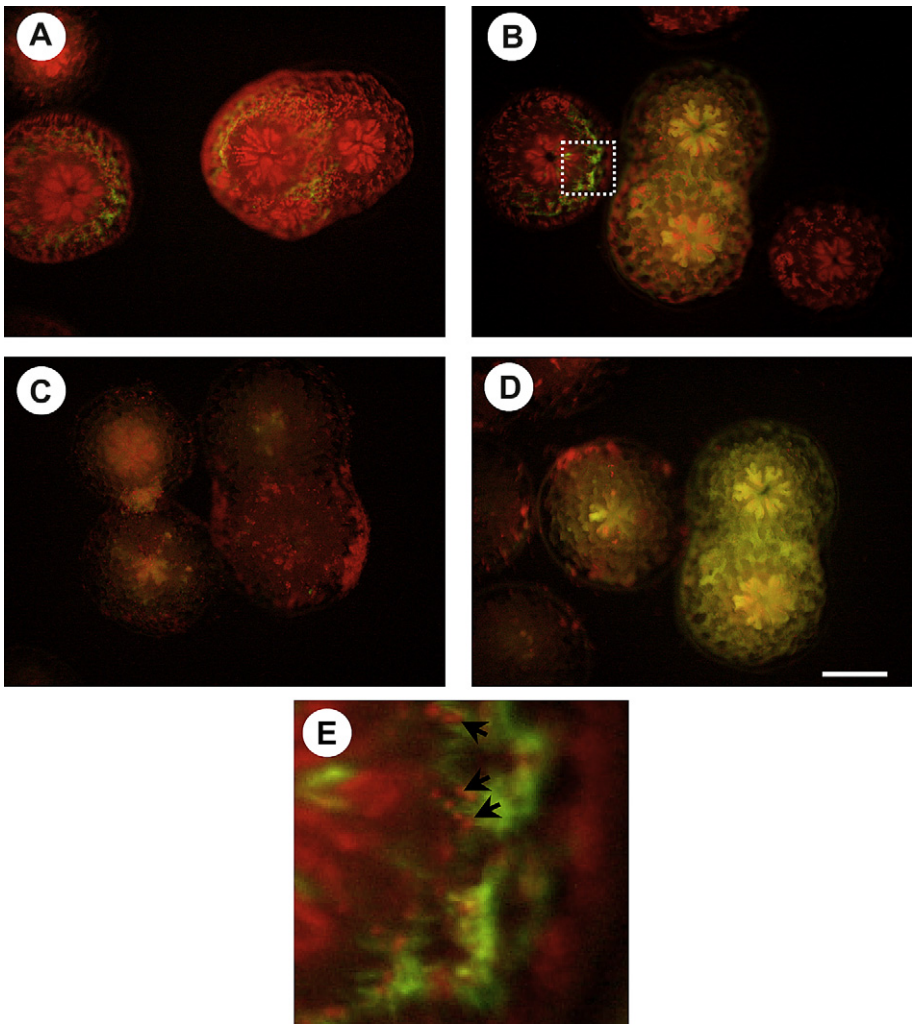

Fig. 2. Fluorescence microscopic images of *Acropora tenuis* primary polyps before and after high-temperature treatment. Polyps were associated with clade A *Symbiodinium* (A, C) or clade D *Symbiodinium* (B, D). Polyps were photographed before (A, B) and after (C, D) treatment. Enlarged image of the area surrounded by the broken line is shown in (E). The red dots represent *Symbiodinium* cells (arrows). Some polyps show green fluorescence. Green fluorescence exists around *Symbiodinium* cells in the body wall and tentacles. Scale bar = 0.5 mm.

4.1. Expression of stress-related genes of juveniles associated with clade A or D symbionts

GFP is a stress-response protein in corals (Smith-Keune and Dove, 2007). For example, Smith-Keune and Dove (2007) showed the downregulation of a GFP-homolog of *A. millepora* adult colonies at 32 °C and 33 °C. They suggested that the GFP-homolog can be used as a sensitive marker of heat stress given that the decreased gene expression preceded damage of the photosystem II of algal symbionts, as measured by PAM fluorometry. In addition, Rodriguez-Lanetty et al. (2009) reported that in aposymbiotic larvae of *A. millepora*, the expression of a fluorescent protein homolog, DsRed-type FP, decreased in response to elevated temperature. They further suggested that DsRed-type FP and the colorless GFP-homolog reported by Smith-Keune and Dove (2007) might be the same. However, we found that the expression patterns of GFP and the RFP-homolog changed in the opposite direction when juveniles associated with different *Symbiodinium* types were exposed to thermal stress. Despite the lack of biological replicates in this study, the present results suggest that the stress response of the coral is not unidirectional, but that it changes direction depending on the symbiont type. Quantification of the GFP content within the juveniles also revealed that the GFP content increased in response to thermal stress in clade D symbionts harboring juveniles, but not in clade A symbionts harboring juveniles.

We also found that the expression of genes encoding an oxidative stress-responsive protein and SLC23 was downregulated in juveniles associated with clade A symbionts, while it was upregulated in juveniles associated with clade D symbionts (Fig. 4B, C). The oxidative stress-responsive protein and the Na^{+}-dependent ascorbic acid transporter family SLC23 (Takanaga et al., 2004) are potential candidates for stress markers in corals. Ascorbic acid is an effective antioxidant and an essential cofactor in numerous enzymatic reactions. Ascorbic acid peroxidase plays a central role in the ascorbic acid/glutathione cycle and is a key enzyme in the reduction of cellular reactive oxygen species (ROS). Conversely, other symbiosis-related genes such as those encoding lipase and sulfate transporter were downregulated by thermal stress in juveniles associated with either clade A or clade D symbionts (data not shown). Thus, not all stress-responsive genes showed bidirectional changes depending on the symbiont type.

Although the present tendency of gene expression patterns was obtained using a few hundreds of juveniles, further experiments using juveniles from different parents are necessary to confirm the

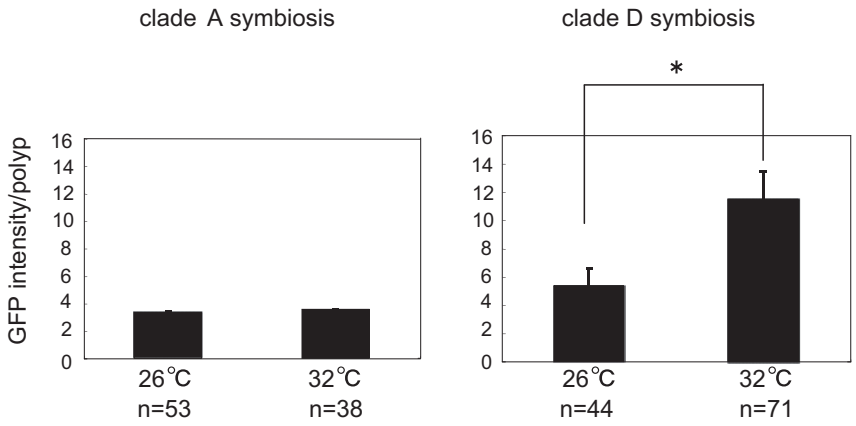

Fig. 3. GFP contents estimated from fluorescence image analyses. Bars represent the mean GFP fluorescence intensities for polyps kept at 26 °C or 32 °C. *Error bars* are standard errors. The asterisk indicates a significant difference ($P < 0.05$).

results. The tendency can be applied only to the coral with a horizontal mode of symbiont transmission and further studies are necessary to understand whether symbiont type also affects host stress response in corals with a vertical mode of symbiont transmission.

4.2. Possible function of fluorescent proteins in the stress response

Salih et al. (2000) suggested that FPs play a photoprotective role for symbionts and enhance the resistance to bleaching during periods of heat stress. GFP from the hydromedusa *Aequorea victoria*

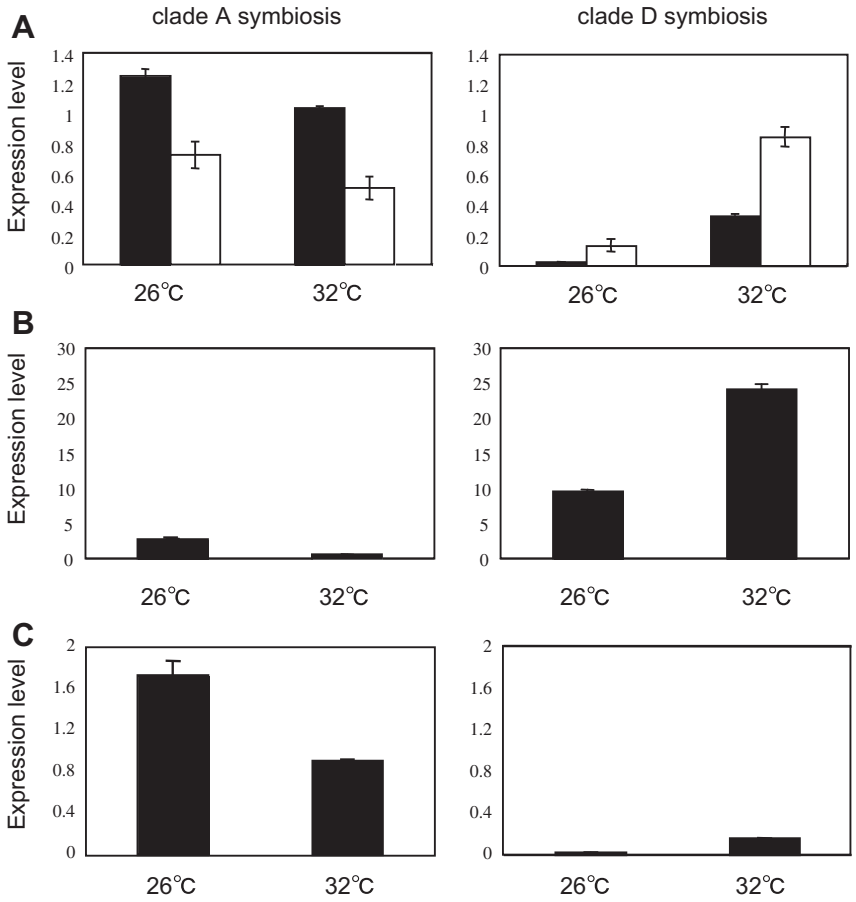

Fig. 4. Results of the quantitative real-time PCR analysis using coral juveniles associated with clade A or clade D *Symbiodinium*. (A) Expression of the green fluorescent protein-homolog (black bar) and red fluorescent protein-homolog (white bar) coding genes at 26 °C and 32 °C. (B) Result for the SLC23-homolog. (C) Result for the oxidative stress-responsive protein-homolog. The expression of these genes is downregulated in high temperature (32 °C) in juveniles associated with clade A symbiont but upregulated in juveniles associated with clade D symbiont. The means and standard deviations of triplicate reactions using the same total RNA are shown. The *y*-axis indicates the fluorescence intensity relative to the control, ribosomal protein L5.

quenches superoxide radicals (O^{2-}) and exhibits SOD-like activity (Bou-Abdallah et al., 2006). Palmer et al. (2009) showed that coral FPs exhibited significant H_2O_2 scavenging activity. These studies suggest that FPs might have antioxidant activity as well as photoprotective functions. If this is the case, different contents of FPs might imply different tolerance against oxidative stress under high temperature. Thus, different expression patterns of FPs in juveniles associated with clade A and clade D symbionts under thermal stress may result in different stress tolerances in these juveniles. However, the present results showed that algal density decreased under thermal stress (32 °C, 24 h) in both juveniles associated with clade A and clade D symbionts (Fig. 1). Further studies are necessary to clarify how GFP gene expression and GFP content affect the stress tolerance of juveniles of the coral.

In many corals, distinct morphs are found that differ greatly in their concentration of FPs (Dove et al., 2001). We observed that the GFP content was higher in juveniles associated with clade D *Symbiodinium* than those associated with clade A symbionts and that the fluorescence intensity increased significantly at high temperatures in clade D harboring juveniles. These results suggest that the amount of GFP, and hence coral color, might be affected by *Symbiodinium* types as well as environmental conditions.

5. Conclusion

The present study shows that the expression levels of four stress-related genes changed in opposite directions depending on the symbiont types, clade A or D. Specifically, gene expression decreased at high temperatures if juveniles were associated with clade A symbionts but increased if they were associated with clade D symbionts. The direction of change in gene expression may depend on the extent of oxidative stress within the cell, and the intracellular oxidative stress of the host cells may vary depending on the symbiont type even when exposed to the same stress conditions. If this is the case, gene expression might be upregulated at low to moderate stress levels but downregulated under conditions of greater stress. Another possibility is that symbionts of different types modulate host gene expression differently by some unknown mechanism. The present results were obtained using juveniles of a coral and further studies are needed to understand how symbiont type affects stress response of adult colonies in the field. The present study highlights the possibility that changes in the expression of stress marker genes may be bidirectional depending on the symbiont type of the coral.

Acknowledgments

We are grateful to staff of Sesoko Station, Tropical Biosphere Research Center, University of the Ryukyus especially Mr. Yoshikatsu Nakano for supporting facility use and to late Dr. Toshiki Wananabe, the University of Tokyo, Ocean Research Institute, for the invariable support. We thank to Dr. Mitsunori Ishihara at National Institute for Environmental Studies and Dr. Tomihiko Higuchi at the Shizuoka University for supporting image analysis of fluorescent protein. This work was supported by Grant-in-Aid for Scientific Research on Innovative Areas 'Coral reef science for symbiosis and coexistence of human and ecosystem under combined stresses' (# 20121002) from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) Japan.

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